The Spatial and Temporal Expression of Ch-en, the engrailed Gene in the Polychaete Chaetopterus, Does Not Support a Role in Body Axis Segmentation

Elaine C. Seaver,* 1 David A. Paulson,* Steve Q. Irvine,† and Mark Q. Martindale*

*Kewalo Marine Lab, PBRC/University of Hawaii, 41 Ahui Street, Honolulu, Hawaii 96813; and †Department of Molecular, Cellular and Developmental Biology, Yale University, KBT 1030, 266 Whitney Street, New Haven, Connecticut 06511

We are interested in understanding whether the annelids and arthropods shared a common segmented ancestor and have approached this question by characterizing the expression pattern of the segment polarity gene engrailed (en) in a basal annelid, the polychaete Chaetopterus. We have isolated an en gene, Ch-en, from a Chaetopterus cDNA library. Genomic Southern blotting suggests that this is the only en class gene in this animal. The predicted protein sequence of the 1.2-kb cDNA clone contains all five domains characteristic of en proteins in other taxa, including the en class homeobox. Whole-mount in situ hybridization reveals that Ch-en is expressed throughout larval life in a complex spatial and temporal pattern. The Ch-en transcript is initially detected in a small number of neurons associated with the apical organ and in the posterior portion of the prototrochophore. At later stages, Ch-en is expressed in distinct patterns in the three segmented body regions (A, B, and C) of Chaetopterus. In all segments, Ch-en is expressed in a small set of segmentally iterated cells in the CNS. In the A region, Ch-en is also expressed in a small group of mesodermal cells at the base of the chaetal sacs. In the B region, Ch-en is initially expressed broadly in the mesoderm that then resolves into one band/segment coincident with morphological segmentation. The mesodermal expression in the B region is located in the anterior region of each segment, as defined by the position of ganglia in the ventral nerve cord, and is involved in the morphogenesis of segment-specific feeding structures late in larval life. We observe banded mesodermal and ectodermal staining in an anterior–posterior sequence in the C region. We do not observe a segment polarity pattern of expression of Ch-en in the ectoderm, as is observed in arthropods. © 2001 Academic Press

Key Words: segmentation; annelid; polychaete; Chaetopterus; engrailed; CNS; mesoderm.

INTRODUCTION

A common design feature of multicellular animals is the serial repetition of body parts along the anterior–posterior axis. In three of the largest and most diverse phyla, the arthropods, annelids, and chordates, these reiterated structures are further elaborated into discreet body segments. It is currently unknown whether the segments of annelids, arthropods, and chordates evolved independently or if their last common ancestor possessed a segmented body plan. It has been proposed by various researchers that segmentation evolved once, twice, or three separate times (reviewed in Davis and Patel, 1999). Until recently, the two major overtly segmented protostome phyla, the annelids and arthropods, had been grouped together as sister taxa in the Articulata and thus assumed to share a common segmented ancestor. Recent evidence suggests that annelids and arthropods may be contained within separate clades, the Lophotrochozoa and the Ecdysozoa, respectively (Aguinaldo et al., 1997; de Rosa et al., 1999). Both the Lophotrochozoa and the Ecdysozoa contain many unsegmented phyla, suggesting that segmentation either arose independently or that overt body-plan segmentation was lost mul-

* To whom correspondence should be addressed. Fax: (808) 599-4817. E-mail: seaver@hawaii.edu.
multiple times in the Metazoa. One way of addressing the evolutionary ancestry of body-plan organization is to understand the cellular and developmental mechanisms leading to the origin of individual body segments. A great deal is known about this in a few representative arthropods, and if segmentation evolved only once in a protostome ancestor, the ontogenetic mechanisms should be largely shared between the Lophotrochozoa and the Ecdysozoa.

The process of segment formation has been best studied within the arthropods, and it is clear that, at both the morphological and the molecular level, there is significant variation within this group. For example, in the long germ band insect Drosophila, all segments are established within a limited temporal window in an acellular environment. By contrast, in grasshoppers, segments form in a cellularized context in a progressive manner in a roughly anterior-to-posterior progression. In most crustaceans, the majority of body segments are added postembryonically. Similarly, several molecular components of the segmentation pathway appear not to be conserved even within insects (reviewed in Nagy, 1994), although recent data from spiders suggest that the ancestral insect utilized representatives of both the segment polarity and pair-rule genes in generating a segmented body plan (Damen et al., 2000). There is one molecular component of the segmentation pathway that has a well-conserved expression pattern in all arthropods.

FIG. 1. Diagrammatic representation of several stages of Chaetopterus larval development (after Irvine et al., 1999). Anterior is to the left in all panels. Stages L1–L7 are shown in lateral views with ventral down. The adult is a dorsal view. Segments are labeled for each body region (A, B, and C) at stages at which they are externally morphologically visible. A more detailed description of the developmental stages is presented in Irvine et al. (1999). Afo, accessory feeding organ; amt, anterior mesotroch; an, aliform notopodium; at, apical tuft; bl, blastopore; gs, gametogenic segments; mt, mesotroch; nr, notopodial rudiment; pal, palette; pmt, posterior mesotroch; pyg, pygidium.
examined, regardless of life history habitat and body-plan specialization. This is the segment polarity gene en (en), a transcription factor whose function has been characterized in detail in Drosophila. It is initially expressed in stripes in the posterior compartment of the segment primordia of the ectoderm and transiently in mesoderm. En acts as an initiator of a signaling pathway that is causally involved with the establishment of differences within each body segment along the anterior–posterior axis (DiNardo et al., 1985; Lawrence, 1992). At later stages, en is also expressed in other tissues such as the forming imaginal discs and in a subset of neurons in the central nervous system. The fact that en has a highly conserved expression pattern in the large number of arthropods examined makes it a useful molecular character for comparisons with annelids.

Within the annelids, the expression pattern of en has been characterized in the leech embryo and it is expressed in a subset of cells in the segment primordia in both the mesodermal and ectodermal lineages (Lans et al., 1993; Wedeen and Weisblat, 1991). The earliest expression is observed in the O and P lineages, when the segment primordia of each lineage has six and five cells, respectively, suggesting that en is part of a signaling pathway that patterns segments in the leech embryo. However, single-cell laser ablations of the en-expressing cell or its precursor in both the O and P lineage result in normal patterning of the remaining segmental clone (Seaver and Shankland, 2001). In addition, ablations of en-expressing cells in the N lineage do not result in any defects in separation of the nervous system primordia into discrete ganglia of the CNS (Shain et al., 1998). These results provide no evidence for a role of en in segmental patterning of the leech embryo and suggest that the establishment of segment polarity does not require cell–cell interactions. Leeches have many derived characters that make it hard to predict whether the mecha-
isms by which they generate segments can be generalized for annelids. It is therefore critical to extend our studies of segmentation to include additional annelid taxa before comparisons of segment formation can be made with other clades, such as arthropods. Although the annelids are almost certainly paraphyletic (reviewed by McHugh, 2000), the polychaetes are the largest and most diverse group within the annelids and generally considered to be basal to the clitellates, which include the oligochaetes and leeches (Westheide, 1997).

We have studied the expression of en in the segmented spionid polychaete worm Chaetopterus (Fig. 1) (Rouse and Fauchald, 1997). If the annelids and arthropods share a common segmented ancestor, it is possible that en will be expressed in a restricted portion of the segment primordia prior to morphological segmentation. We have isolated a cDNA clone representing a single member of the en class of homeodomain-containing class of transcription factors and studied the spatial distribution of its transcript during larval development. We have found that Ch-en is expressed in a complex and dynamic manner in all stages of larval life, but it is not expressed in ectodermal stripes or in the posterior boundary of segment primordia. Rather, it is expressed in small sets of segmentally reiterated cells in the nervous system and in the elaboration of mesodermal derivatives.

MATERIALS AND METHODS

Animals

Adult Chaetopterus were obtained from Marine Resources (Woods Hole, MA) and used to establish laboratory larval cultures according to Irvine and Martindale (1999). Larval staging and nomenclature are as described by Irvine et al. (1999).

En cDNA Clone Isolation and Library Screening

A previously generated larval Chaetopterus cDNA library (Irvine and Martindale, 2000) was used as a template in a PCR reaction to amplify a 220-bp fragment with primers designed against conserved regions of the en gene, including the homeobox domain. The sequences of the primers are as follows: en-1, 5'-GACAAGCCGRCDD-MGVACVGNCNTT-3'; en-2, 5'-TGRTRTANARNCYTNGC-CATC-3'. The PCR fragment was radiolabeled with [32P]dCTP (Amersham) by using the Megaprime DNA labeling system (Amersham) and used as a probe to screen a lambda gt10 larval cDNA library (Irvine and Martindale, 1999). A total of 4 × 105 plaques were screened on duplicate filters by hybridizing at 65°C in 5× SSC, 5× Denhardt's solution, 1% SDS, and 100 μg/ml salmon sperm DNA (Sigma). The blot was then washed at room temperature in a series of increasingly stringent washes: 2× SSC, 0.1% SDS; 2× SSC, 0.1% SDS; and a final high-stringency wash: 0.1× SSC, 0.1% SDS at 68°C, and exposed to film.

Southern Analysis

Sperm was collected from reproductive parapodia in gravid adults, and genomic DNA was extracted from sperm by using DNeasy (Molecular Research Center) and 100 μg/ml proteinase K (GIBCO/BRL). DNA (5 μg) was digested overnight in separate reactions with the following restriction enzymes: EcoRV, HpaI, DraI, SacI, and SphI (New England Biolabs). Digested DNA was size fractionated by electrophoresis through a 0.7% agarose gel (TAE buffer), depurinated with a 30-min exposure to 0.25 N HCl, and transferred to a positively charged nylon membrane (S & S Nytron Supercharge, Schleicher and Schuell) using alkaline conditions (3 M NaCl, 8 mM NaOH). The 220-bp fragment used to screen the Chaetopterus cDNA library was radiolabeled with [32P]dCTP (Amersham) with the Megaprime DNA labeling system (Amersham) and used as a probe. Hybridization was performed at 68°C overnight in 5× SSC, 5× Denhardt's solution, 1% SDS, and 100 μg/ml salmon sperm DNA (Sigma). The blot was then washed at room temperature in a series of increasingly stringent washes: 2× SSC, 0.1% SDS; 2× SSC, 0.1% SDS; and a final high-stringency wash: 0.1× SSC, 0.1% SDS at 68°C, and exposed to film.

Fixation and Whole-Mount in Situ Hybridization

Chaetopterus larvae were relaxed for 2–3 min in 0.1% PPOX (propylene phenoxytol) in seawater and fixed in 5% formaldehyde in modified van Loon fix buffer (125 mM Hepes, 2.5 mM MgSO4, 1.25 mM EGTA, pH 6.9, 0.03% Tween 20) preheated to 50°C. Larvae were then fixed for 20 min at room temperature (r.t.) and washed in 1/2 vol of methanol followed by 5-min washes in the following solutions: ME (100 mM EGTA in methanol); 30% ME, 70% FPTw (4% formaldehyde in PBS, pH 7.0, 0.1% Tween 20); 50% ME; 50% FPTw; 30% ME, 70% FPTw. The larvae were then incubated in FPTw for 20 min, dehydrated in methanol, and stored in 100% methanol at −20°C. Rehydration was achieved by 5-min washes in 60% Methol/PTw (PBS, 0.1% Tween); 30% Methol/PTw; 4× 100% PTw. A 2- to 10-min digestion with proteinase K (0.01 mg/ml PTw) was followed by a 1-h fixation in 3.7% formaldehyde in PTw, five washes in PTw, and then heated to 80°C for 15 min to inactivate endogenous phosphatase activity. Larvae were then washed in hybridization solution (50% formamide, 5× SSC, 50 μg/ml heparin, 0.1% Tween 20, 1% SDS, 100 μg/ml salmon sperm DNA) for 10 min at r.t. and then prehybridized in fresh hybridization solution at 65°C for 1–4 h. DIGoxigenin-labeled riboprobes were generated for the 1.2-kb en engaged cDNA clone in both the sense and antisense directions in an in vitro transcription reaction using the MEGAscript kit (Ambion). Hybridization was carried out for 18–24 h at 65°C by using a probe concentration of 0.1 ng/μl. Larvae were washed in the following series: once for 5 min in hybridization buffer (hyb), once for 20 min in hyb, two times for 20 min in 75% hyb, 25% PTw at 60°C, two times for 20 min in 50% hyb, 50% PTw at r.t., two times for 20 min in 25% hyb, 75% PTw at r.t., two times for 20 min in 100% PTw at r.t., and five times in PBT (PBS, 0.2% Triton, 0.1% BSA). The probe was visualized by placing larvae in blocking buffer (Boehringer-Mannheim No. 1096176) for 1 h at r.t. and then exposed to anti-DIG Abs (Boehringer-Mannheim) at a 1:2500 dilution overnight at 4°C. Larvae were then washed five times for 10 min in PBT, three times for 5 min in AP buffer (100 mM NaCl, 5 mM MgCl2, 100 mM Tris, pH 9.5, 0.5% Tween 20). Staining was performed in AP buffer with the addition of 4.4 μl/ml NBT (Amersham, stock: 75 mg/ml in 70% dimethyl formamide) and 3.3 μl/ml BCIP (Amersham; stock: 50 mg/ml in dimethyl formamide).
FIG. 3. Predicted engrailed protein domains from Chaetopterus and alignments showing conservation with en domains from other species. There are five en-specific domains designated EH1-EH5. EH4 contains the homeodomain. Amino acids identical to the Chaetopterus sequence are shown as dashed lines. Abbreviations for en sequences are as follows: Oryzias, Oryzias latipes (AF112141); Danio, Danio rerio en-2 (X68151); Gallus, Gallus gallus en-2 (L12697); H. sapiens en-2 (E48423); Xenopus, Xenopus laevis en-2 (X62973); Musculus, Mus musculus en-1 (A48423); Branchiostoma floridae (U82487); Ctenodrilus, Ctenodrilus serratus (U82487); Periplaneta americana Pa-en1 (A|43883); Anopheles, Anopheles gambiae (U42429); Tribolium, Tribolium castanemum (S73225); Drosophila, Drosophila melanogaster (M10017). Accession numbers are written in parentheses.

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revealed five domains of sequence conservation in the en family: the homeodomain (EH4) and four other domains. Database searches with the predicted amino acid sequence of the Ch-en gene contain all five domains (boxed in Fig. 2). The five domains are well conserved across taxa, including examples from vertebrates and insects (Fig. 3). This is the first report to include sequence data for EH1, EH2, and EH3 for a member of the Lophotrochozoa clade. The Ch-en homeodomain has approximately 80–85% identity with en homeodomains from many species across taxa (Fig. 3), and amino acids shown to be critical for DNA binding are conserved within the Ch-en homeodomain (Kissinger et al., 1990). The homeodomain also contains a conserved region in the third helix that has been suggested to be sufficient for directing the internalization and secretion of the En protein (Joliot et al., 1997, 1998), a pathway independent of the mass transport system of the cell. EH1 is an amino acid in length and has been demonstrated to be involved in repression of transcription (Smith and Jaynes, 1996). EH2 spans 18 amino acids and is the most highly conserved of the en domains (Fig. 3). Downstream and immediately adjacent to EH2 lies the conserved 8 amino acids of EH3. EH2 and EH3 have been shown to be necessary for binding to the Extradenticle/Pbx proteins (Peltenburg and Murre, 1996). EH5 consists of 20 amino acids adjacent to the 3' end of the homeodomain and also contributes to repression of transcription activity (Smith and Jaynes, 1996). In addition, in several en proteins, a proline-rich region towards the 5' end of the protein has been suggested to be an activation domain (Logan et al., 1992). The Ch-en protein contains five prolines 5' of the EH1 domain which may promote activation.

Results

Isolation and Characterization of the Chaetopterus en cDNA

To characterize the engrailed gene from Chaetopterus, we isolated several positive clones from a Chaetopterus larval cDNA library (Irvine and Martindale, 2000) using as a probe a 220-bp PCR fragment which encodes the conserved homeodomain (see Materials and Methods). All clones contained inserts of approximately 1.3 kb. Figure 2 depicts the nucleotide sequence of the clone and its predicted amino acid sequence. The cDNA clone is 1256 nt in length and contains an open reading frame of 960 nt. Because there is a continuous open reading frame through the 5' end of the clone, it is probable that the clone does not contain the initiator methionine. The first in-frame termination codon occurs at nt position 787 and is followed by approximately 470 bp of 3' untranslated sequence. There is a consensus polyadenylation site at nt position 960.

Database searches with the predicted amino acid sequence reveal that the isolated clone from Chaetopterus encodes an engrailed class gene, Ch-en. Amino acid sequence comparisons among en genes from many species (Ekker et al., 1992; Hui et al., 1992; Logan et al., 1992) have revealed five domains of sequence conservation in the en family: the homeodomain (EH4) and four other domains designated EH1, EH2, EH3, and EH5. The predicted amino acid sequence of the Ch-en gene contains all five domains (boxed in Fig. 2). The five domains are well conserved across taxa, including examples from vertebrates and insects (Fig. 3). This is the first report to include sequence data for EH1, EH2, and EH3 for a member of the Lophotrochozoa clade. The Ch-en homeodomain has approximately 80–85% identity with en homeodomains from many species across taxa (Fig. 3), and amino acids shown to be critical for DNA binding are conserved within the Ch-en homeodomain (Kissinger et al., 1990). The homeodomain also contains a conserved region in the third helix that has been suggested to be sufficient for directing the internalization and secretion of the En protein (Joliot et al., 1997, 1998), a pathway independent of the mass transport system of the cell. EH1 is an amino acid in length and has been demonstrated to be involved in repression of transcription (Smith and Jaynes, 1996). EH2 spans 18 amino acids and is the most highly conserved of the en domains (Fig. 3). Downstream and immediately adjacent to EH2 lies the conserved 8 amino acids of EH3. EH2 and EH3 have been shown to be necessary for binding to the Extradenticle/Pbx proteins (Peltenburg and Murre, 1996). EH5 consists of 20 amino acids adjacent to the 3' end of the homeodomain and also contributes to repression of transcription activity (Smith and Jaynes, 1996). In addition, in several en proteins, a proline-rich region towards the 5' end of the protein has been suggested to be an activation domain (Logan et al., 1992). The Ch-en protein contains five prolines 5' of the EH1 domain which may promote activation.

Southern Analysis

To analyze the number of en genes present in the Chaetopterus genome, Southern blots with Chaetopterus genomic DNA were performed using the 220-bp fragment for screening the cDNA library as a probe. This 220-bp fragment encompasses the homeodomain and a portion of domain 5 (EH5). Digestion of genomic DNA by 5 different restriction enzymes yielded a single band in 4/5 digests (Fig. 4) when hybridized with the 220-bp fragment under stringent conditions. In addition, digestion with an enzyme (EcoRI) in which a single restriction site is contained within the 220-bp fragment yields an additional band as expected (data not shown). This suggests that, in Chaetopterus, there is a single copy of the en gene, Ch-en.

Developmental Expression of the Chaetopterus en Gene

We examined the expression pattern of the Ch-en transcript by in situ hybridization of whole-mount animals with a digoxigenin-labeled Ch-en riboprobe. We studied expression for all larval stages of Chaetopterus. The larva hatches after approximately 18 h as a simple swimming gastrula prototrochophore. Chaetopterus has an extended larval life of up to 60 days, and it is during this time that the
adult body plan is formed. A summary of larval stages is shown in Fig. 1. The adult body is divided into three major regions: the A, B, and C regions. All three regions are segmented, although there is not an anterior-to-posterior temporal progression in the appearance of morphological segmentation; the B region generates intersegmental clefts which represent the external morphological manifestation of segments before the A region. During larval life, the A and B regions undergo extensive morphogenesis. The C region contains the gametogenic segments, and only a few segments in this region are formed prior to metamorphosis. Larval development has been divided into seven stages (L1–L7) by Irvine and Martindale (1999), and we use the same nomenclature here. At hatching, the L1 larvae have very little morphologically differentiated tissue; its most notable feature is the apical tuft. Within approximately 36 h of hatching (L2), the basic body plan is visible. L3 is characterized by the appearance of the mesotroch, the first ciliary band to form (Fig. 1). L4 is the first stage in which morphological segmentation is apparent, occurring in the five segments of the B region. Overt segmentation in the 10 segments of the A region appears in the following stage, at L5. L6 represents midmetamorphosis in the transformation into the juvenile (L7).

Early Larval Stages

The earliest stage of larval development at which we could detect the en transcript message is in L1. Initially, the transcript is restricted to two bilaterally symmetric cells in the posterior region of the prototrochophore (Fig. 5A). Slightly later, the transcript is present in four cells (or patches) in the posterior half of the animal. In addition, there is staining in approximately three cells associated with the apical tuft (Fig. 5B). In late L1 larva, we observed a labeled cell on the dorsal side, at approximately the same axial level as the mouth (not shown). We believe that these early en-expressing cells are neurons based on their position and the fact that we observed these cells in the process of delaminating inward from the ectoderm towards the interior of the larvae (asterisks). In addition, approximately three cells express en in the region of the apical tuft (arrowhead).

Midlarval Stages

Early, during the L3 stage, the mesotroch appears, which marks the boundary between the A and B regions. Ch-en is expressed posterior to this ciliary band in the B region (Figs. 6A and 6B). It is initially expressed laterally in two small patches of cells in the ectoderm (Fig. 6C). The labeling expands to form two crescents predominantly in the mesoderm around the circumference of the body with a notable absence of labeling at both the dorsal and ventral midlines (Figs. 6B and 6D). There is no labeling in any other region of the larvae except for a single cell on the dorsal side of the head, possibly a neuron (data not shown). At late L3, staining appears in the A region as approximately two to three bilaterally symmetric patches of cells straddling the ventral midline at the edge of the developing CNS (data not shown).

At the L4 stage, there is staining in all three body regions. The patches of ectodermally derived cells straddling the ventral midline of the A region expand to 10 bilaterally symmetric patches of Ch-en staining (Fig. 7A). These patches correspond with the future 10 segments of the A region, and each is located at the lateral edge of the CNS.
and at the boundary between adjacent ganglia. By L4, the ganglia are well formed and axon tracts are present in the ventral nerve cord (Irvine et al., 1999). The arrangement of the en-positive cells approximating a V shape reflects the modified nervous system in the A region of Chaetopterus (Martin and Anctil, 1984). The patches of Ch-en labeling in the CNS appear prior to the overt external morphological segmentation of the A region. The labeling associated with the anterior mesotroch is distinct from the other staining in the A region in that there is patchy labeling under the surface epithelium immediately posterior to the anterior mesotroch that spreads circumferentially (Fig. 7A). Although we are not certain of the identity of these cells, they are located subepidermally, and we propose that they are either peripheral neurons innervating the anterior mesotroch or scattered mesodermal cells.

In the B region during L4, there is Ch-en staining in both the CNS and the mesoderm. The broad mesodermal band of staining present during L3 splits into five bands, corresponding to the five B segments that form during this stage (Fig. 7B). The transition of staining from a broad region into five distinct bands is coincident with morphological segmentation. We never saw the banded staining pattern of Ch-en prior to morphological segmentation. As they mature, the Ch-en bands in B3–B5 extend from the ventral side dorsally (Fig. 7C), but are absent from both the dorsal and ventral midlines (Figs. 7A and 7D). The band of staining in the B2 segment is more restricted to the ventral side of the larvae (Fig. 7C), and this segment has a distinct morphology relative to the other B segments. Ch-en staining in the B1 segment is also distinct from the staining in other B segments. Most notably, there is labeling that extends to the dorsal midline (Fig. 7C); the aliform notopodia will form on the dorsal surface of B1 at later stages of development (see below).

During L4, ectodermal staining is restricted to the CNS (Figs. 7A, 7D, 7E, 8B, and 8C). In the B region, labeling is present at the lateral edge of the ganglia in a position similar to that seen in the A regions (Figs. 7D, 7E, 8B, and 8C). In the B region, however, the staining extends more laterally from the CNS in the position of the segmental nerve (Figs. 7D and 7E) (Irvine et al., 1999).

A bilaterally symmetric patch of staining is present in the C region during L4 in both the mesoderm and the CNS (Figs. 7B and 7D). The labeling is very similar to that seen in B3–B5, but more restricted to the ventral portion of the larvae. The labeling is associated with C1, which, at this stage, is morphologically visible as a distinct segment (Fig. 7D). We consistently observed nascent segments posterior to C1 that do not have any Ch-en staining (Fig. 7D). The unlabeled segments are smaller in width and length and less differentiated than the C1 segment. We did not observe Ch-en labeling in the C region prior to morphological segmentation. By late L4, we often observed two patches of labeling in the C region, corresponding to the segments C1 and C2 (Fig. 7C).

At L5, the larvae are competent to undergo metamorpho-
sis and external morphological segmentation in the A region is now readily apparent: the chaetal sacs are visible and the distal tips of the chaete extend past the body wall. The distribution of the Ch-en transcript in L5 is very similar to that of L4 with maturation of the existing pattern. In the A region, the patches of Ch-en staining elongate mediolaterally from their previous smaller more rounded appearance in L4 (Fig. 8A). We do not know if this change is due to cell proliferation or movement of cells. In addition, there is labeling associated with a small number of mesodermal cells at the base of the developing chaetal sacs in the A region, which are located beneath the CNS. The mesodermal labeling associated with the posterior edge of the anterior mesotroch becomes more prominent and extends dorsally (data not shown). The dorsal staining in B1 becomes more extensive, expanding ventro-laterally from the dorsal midline. The mesoderm staining in the segments B3–B5 extends dorsally although never reaches the dorsal midline (not shown). In the C region, there are two patches of labeling (Fig. 8B) very similar to the pattern observed in late L4 (see Fig. 7C) present in the ventro-lateral portions of the segments C1 and C2 in the mesoderm and CNS. The Ch-en transcript is restricted within the segment along the anterior–posterior axis (Figs. 7 and 8). In the B region, the mesodermal staining is located anterior to the corresponding CNS staining for each B segment (Fig. 8B). Using the ganglia of the CNS to define the position of the segmental boundaries, the anterior boundary of the mesodermal staining is equivalent to the anterior boundary of the segment (Fig. 8B). Furthermore, the external morphological grooves ("valleys") correspond to a region where the peripheral roots project, just posterior to the ganglionic midpoint, and do not correspond to interganglionic positions.

**Late Larval Stages**

In L6, the larvae are in midmetamorphosis. The A region becomes flattened in the dorso-ventral axis, and the parapodial rudiments extend laterally. There is no longer any labeling in the A region, neither in the CNS nor associated with the chaetal sacs (Fig. 9A). In the B region, the banded labeling in the mesoderm of setigers B3–B5 persists but is less prominent than during L5 (Fig. 9A). The most prominent labeling at this stage is in segment B1 (Figs. 9B and 9C). The bilaterally symmetric patches straddling the dorsal midline have extended ventro-laterally (Fig. 9B) to meet a lateral patch of staining approximately halfway between the dorsal and ventral faces of the larvae (Fig. 9C). This labeling is associated with morphogenesis of a pair of modified parapodia on the dorsal side called the aliform notopodia, used in feeding. The lateral patches correspond to the future distal tip of the aliform notopodia. This pattern of Ch-en staining appears before the aliform notopodia protrude from the body wall.

L7 represents the postmetamorphic juvenile. Prominent staining from previous stages, such as in the CNS in the A region and in the mesoderm in the palettes of B3–B5, has disappeared by this stage (Fig. 10A). Ch-en staining is most prominent in the mesodermal component of newly forming structures, the aliform notopodia in B1 and the accessory feeding organ in B2 (Fig. 10B). The aliform notopodia has extended out from the body wall by this stage, and there is intense Ch-en labeling at the distal tip in the mesoderm (Figs. 10B and 10C), with lighter labeling in more proximal portions. In addition, there is labeling on the dorsal face of the setiger B2 in the position of the accessory feeding organ (Fig. 10B). This structure forms a groove along the anterior–posterior axis. Its function remains unclear, but it has been proposed to be involved in feeding (Enders, 1909). In L6, the staining in this region of B2 is perpendicular to the body axis, and over time it progressively reorients, becoming two lines running parallel to the anterior–posterior axis, reflecting the morphology of the mature canal. The other notable area of staining during L7 is in two bilaterally symmetric patches in the C region at the posterior portion of the forming parapodia of C1 and C2 (Fig. 10D). These patches of staining persist from L4 (Fig. 7C).

**DISCUSSION**

We have isolated and characterized an en gene, Ch-en, from the polychaete annelid Chaetopterus, the first authentic report of the spatial and temporal expression of an engrailed transcript in any polychaete. The protein sequence encoded by the Ch-en gene contains an en-class homeodomain and four additional domains well conserved among members of the en family. Sequence analysis of these domains reveals that none of the Ch-en domains shares more amino acid identity with arthropod en domains than with vertebrate en domains or vice versa. The Ch-en gene does not contain the RS dipeptide between the EH2 and EH3 domains present in the insect gene in Drosophila and in other arthropods that have a single en gene such as Schistocerca (Patel et al., 1989b) and Artemia (Manzanares et al., 1993). Genomic Southern blot analysis using high-stringency conditions supports the conclusion that there is a single en in Chaetopterus, although we cannot rule out the presence of another gene until a thorough genomic analysis has been performed. Our results are consistent with the hypothesis that there was only a single ancestral en-class gene in the eumetazoan ancestor and that en has independently duplicated in several lineages, including higher insects (Coleman et al., 1987; Hui et al., 1992), vertebrates (Joyner and Martin, 1987), barnacles (Gilbert et al., 2000), a cephalopod mollusc (Wray et al., 1995), and the apyrgote insect Thermobia domestica (Peterson et al., 1998). Currently, there are not enough sequence data outside the homeodomain available for en from other members of the Lophotrochozoa to perform a phylogenetic analysis of this gene.

The Chaetopterus body is composed of a large number of individual body segments subdivided into tagmatised body regions. Furthermore, Chaetopterus exhibits heterochrony.
in the morphological delineation of segments along the anterior-posterior axis: the five segments of the B region appear prior to the anterior-most segments (Irvine et al., 1999). This modification from a more common polychaete form that has a progressive anterior to posterior addition of body segments may reflect an adaption to particular life history characteristics. Selective pressure may have resulted in an acceleration of the differentiation of the B region segments, which are critical for feeding in Chaetopterus. It is not currently known whether changes in the relative timing of appearance of segments also represent a modification in the basic mechanism by which the segments are formed. Although sequential addition of segments such as that seen in the C region is often associated with generation of segments by teloblastic growth, it may be that all the segments of Chaetopterus are generated by a similar mechanism and that temporal changes can account for the modifications in the development of Chaetopterus body segments. Future experiments must be performed to test such an hypothesis.

The transcript of the Ch-en gene is expressed throughout larval life and in the postmetamorphic juvenile in a dynamic spatial and temporal pattern in both ectodermal and mesodermal derivatives. We have not examined expression during embryogenesis or adult stages; however, embryogenesis lasts only 18 h, and it is during the 60-day larval life span that the segmented adult body plan is formed. Initially, the Ch-en transcript is transiently expressed in a small number of probable neurons in the prototrochophore including cells associated with the apical tuft. Ch-en is also expressed in the nervous system at later stages of larval life in small patches of cells at the lateral boundary of the CNS in segments of all three body regions. Whether these groups of Ch-en-expressing neurons are serially homologous or not will have to be studied using cell lineage techniques; however, it is striking that similar groups of cells expressing this gene reside in similar regions of the ganglia in every body segment. The development of the Chaetopterus CNS has not been described in detail, and therefore it is difficult to pinpoint the exact identity of these cells within the nervous system. From their position, it is likely that these clusters of cells are neurons or glia.

Each of the three larval/adult body regions has a distinct pattern of Ch-en expression, and, furthermore, some individual segments within a single body region have unique expression patterns of en. These differences reflect modifications of segment morphology along the anterior-posterior axis. In addition, the temporal dynamics of expression in the different body regions mirror morphogenesis of particular structures. The 5 segments of the B region, lying posterior to the 10 segments of the A region, are the first to show overt, externally identifiable body segments. Once the larva has morphologically distinct body regions, Ch-en expression is limited to the B region. It is initially expressed as a broad band in the mesoderm, which then resolves into distinct bands, one band/segment, coincident with but not prior to morphological segmentation. The temporal and spatial deployment of Ch-en in the B region mirrors distinct morphogenetic pathways. For example, the segment B1 is the only B segment that has mesodermal Ch-en expression at the dorsal midline, and this expression is associated with morphogenesis of the large aliform notopodia, a structure unique to B1. In addition, the development of segment B2 is delayed with respect to the remaining segments and during larval stages it does not exhibit a differentiated segment morphology. The expression of Ch-en in B2 is likewise reduced relative to that seen in segments B3–B5, the segments that form the large palettes which create water currents in the adult tube. Thus, Ch-en is likely to be involved with the mesodermal morphogenesis of the large palettes in these segments and not with the individualization of body segments per se.

In addition to expression associated with a subset of cells in the CNS, Ch-en is transiently expressed in the A region associated with the iterated structures of the chaetal sacs. This mesodermal expression begins during L5 after the CNS staining but disappears at approximately the same time, just before chaetae emerge laterally as the parapodial rudiments of A1–A10.

In the C region, two bands of Ch-en expression appear in the mesoderm and ectoderm during larval life. The appearance of the labeling is similar to that observed in the B region, but more restricted to the ventral side of the larvae. Ch-en expression appears in the C region in segments C1 and C2 after these segments are morphologically visible. In our observations of postmetamorphosis L7 animals, there are two pairs of parapodia in the C region, consistent with the development of two segments by this larval stage and similar to observations by Enders that two sexual segments form during larval life (Enders, 1909). We did not examine Ch-en expression during adult stages after many segments have been added to the C region, but we would predict that the pattern of expression in more posterior segments is similar to the pattern we observe in the two most anterior segments.

It is interesting to note that, in each of the three body regions, there is a distinct onset of Ch-en expression relative to the appearance of morphological segments. In the A region, Ch-en appears in segmentally iterated cell types prior to morphological segmentation of the A segments; in the B region, a segmentally iterated pattern of Ch-en expression appears coincident with morphological segmentation of the B region; and, in the C region, segments appear prior to the onset of Ch-en in the segments C1 and C2. This suggests that the onset of Ch-en is not temporally linked to morphological segmentation in a consistent manner.

Genetic analysis has shown that en clearly has an important role in the process of body-plan segmentation in flies: mutants have changes in polarity and pattern in the posterior compartment of every segment. The conserved expression pattern of en in other insects and crustaceans supports this role in arthropods. One striking feature of en expression in arthropods is that, regardless of variations in segment morphology, tagmatization patterns, or differences in
FIG. 7. During L4, Ch-en is expressed in all three body regions (A, B, and C). (A) Ventral view (anterior is to the top). The Ch-en transcript is present in 10 bilaterally symmetric patches at the lateral edge of the CNS of the A region (arrowheads), one pair per segment. Immediately posterior to the anterior mesotroch, there are scattered labeled cells (small arrows). Ch-en expression is also visible in the ganglia of the first two segments in the B region. (B) From L3 to L4, the Ch-en transcript resolves from a broad expression to a banded pattern corresponding with the five segments of this region (B1–B5) and a single smaller band in the most anterior segment of the C region (C1). Early L4 stage. Lateral view, anterior is to the left, ventral is down. (C) Higher magnification of a late L4 larva viewed from the lateral side (anterior to the left, ventral down). Ch-en is expressed in the mesoderm and ectoderm (D, E, and Fig. 8) in a segmentally iterated pattern in the B region. At this stage, expression is higher in the ventral portion of the segments B2–B5. In B1, there is expression at the dorsal midline at the future site of the aliform notopodia (arrow). The staining in B2 is less extensive than in other segments of the B region which corresponds to the distinct morphology and delayed morphogenesis in this segment. Arrowheads point to two bands of labeling in the C region in the morphologically visible segments C1 and C2. (D) Ventral view of the B region in a mid-L4 larva; anterior is to the left. Ch-en is expressed at the lateral edge of the ganglia (see also E) in each of the B segments (arrows). The staining is positioned in the “valleys” of each morphological segment and extends a short distance dorsolaterally. Note the lack of expression at the ventral midline. In B2–B5, there is also no expression at the dorsal midline. There is labeling in C1, and immediately posterior, there are two nascent segments which do not express Ch-en (arrowheads). (E) Corresponding view of (D) with the nuclear stain Hoescht dye to show the position of the ganglia of the CNS.
mechanisms of segment formation, en expression at the posterior boundary of the segment is highly conserved within an individual from one segment to another (Patel et al., 1989a,b; Scholtz et al., 1994). This is in contrast with what we observe in Chaetopterus in which there are differences in Ch-en expression reflecting distinct morphologies of particular segments. At no stage or body region do we observe ectodermal stripes in the segment primordia. Another characteristic of en expression in arthropods is that the ectodermal stripe pattern appears prior to overt morphological segmentation. Although we cannot eliminate the possibility that en influences patterning within an already established segment, the transient temporal dynamics of Ch-en expression is more consistent with cell-type differentiation events rather than regional specification within the segment. Taken together, the expression pattern of en during larval stages in Chaetopterus does not resemble the highly conserved pattern in arthropods, and the spatial and temporal dynamics of expression do not obviously support a role for en in segment formation in Chaetopterus.

en orthologs have also been studied in the segmented deuterostome phylum Chordata. In the cephalochordate Amphioxus, en is expressed in mesoderm during somitogenesis (Holland et al., 1997) and en is expressed in a segmentally iterated pattern along the A/P axis in a subset of motorneurons in zebrafish (Hatta et al., 1991). These data have led some workers (for example, Carroll et al., 2001; De Robertis, 1997; Kimmel, 1996) to propose that the common ancestor of protostomes and deuterostomes was segmented and utilized en to generate individual body segments. However, en orthologs are expressed after somitogenesis has already occurred in vertebrates and are only expressed in some of the developing somites in Amphioxus. Furthermore, it is clear that en orthologs have been co-opted for other functions within the vertebrates, such as establishment of the midbrain/hindbrain boundary (reviewed in Joyner, 1996) and in patterning the developing limb bud (Loomis et al., 1996).

More recently, en orthologs have been studied in a broader range of taxa across the Metazoa and they are expressed in a wide variety of tissues. In Onychophora, a segmented group considered to be a sister taxa to the arthropods, en has been reported to be restricted to the mesoderm in a portion of the developing segment (Wedeen et al., 1997). In several molluscs (Jacobs et al., 2000; Moshel

FIG. 8. Expression of the Ch-en transcript in L5. (A) Ventral view (anterior is up) illustrating the persistence of expression in the CNS in the A region which elongates along the medio-lateral axis (arrow pointing to one hemisegment). At this stage, the developing chaetae are visible and Ch-en-expressing cells are located at the base of individual notopodial rudiments which are located deep in the tissue (not shown). (B) Lateral view (anterior is to the left and ventral is down). Distribution of the Ch-en transcript in the B region showing the relative position of the ectoderm staining (short arrow), mesoderm staining (arrowhead), and segment borders in segments B3–B5. Long arrow points to the anterior border of Ch-en staining. Note that the external morphological grooves (“valleys”) do not correspond with the interganglionic regions, but rather to a region where the peripheral roots project, just posterior to the ganglionic midpoint. There are also two bands of staining in the C1 and C2 segments of the C region. (C) Ventral midline view of the B1 segment showing presence of the Ch-en transcript in a small cluster of cells at the lateral edge of the ganglion (arrowheads). Anterior is up. (D) Same view as in (C), showing position of a single ganglion with Dapi staining. The high density of nuclei relative to the surrounding epidermis is characteristic of the CNS.

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FIG. 9. The Ch-en transcript is no longer expressed in the A region during L6 (midmetamorphosis). Anterior is to the left for all panels. (A) Ventral view showing lack of expression in the A region and persistence of expression in the segments of the B and C regions. (B) Dorsal view illustrating that in L6 the most prominent Ch-en labeling is associated with the dorsal midline of the B1 segment (arrows). (C) Lateral view of the same animal as in (B) showing expression extending ventro-laterally in B1 (arrow) to a small patch of labeled cells located laterally (arrowhead) which corresponds to the future distal tip of the aliform notopodia.

FIG. 10. There is limited Ch-en expression in the postmetamorphic juvenile (L7). Anterior is to the left for all panels. (A) Dorsal view showing the Ch-en transcript limited to the developing aliform notopodia in the B1 segment, the accessory feeding organ in B2, and the C region. (B) Detailed view of the aliform notopodia showing prominent labeling at the distal tip (arrows) seen from a dorsal view. There is also labeling along the A/P axis at the dorsal midline in the position of the developing accessory feeding organ (arrowhead). (C) Close-up view of a single arm of the aliform notopodia showing that the Ch-en transcript is expressed in the mesoderm of this structure (arrow). Dorsal view. (D) Ch-en is expressed in two bilaterally symmetric patches at the posterior edge of the rudiments of the parapodia of C1 and C2 (arrowheads). Ventral view. Compare with the later stage of development of the parapodia of C1 and C2 in (A).
et al., 1998), en expression is associated with the developing shell glands. en has also been characterized in several deuterostomes, and, in echinoderms, it is localized to the boundaries between forming skeletal ossicles in the ectoderm (Lowe and Wray, 1997). Several hypotheses have been proposed for the ancestral role of en in the Metazoa, including specification of specific neuronal cell types (Patel et al., 1989b), mesoderm patterning (Lans et al., 1993), and more recently patterning the exoskeleton (Jacobs et al., 2000). Expression in the nervous system has been observed in most/all species for which the distribution of en has been described. Expression in the mesoderm is less widespread but has been described across a broad range of taxa including vertebrates, Amphioxus (Holland et al., 1997), leech (Lans et al., 1993), Onychophora (Wedeen et al., 1997), and Drosophila (reviewed in Lawrence, 1992). Our data on the expression of Ch-en support both the neurogenic and the mesoderm hypotheses since it is prominently expressed in both tissues during larval development.

This study was performed to determine whether Ch-en has a role in segment formation in polychaete annelids in order to infer something about the segmental character of the common ancestor of annelids and arthropods. It is obvious that, in Chaetopterus, en is not expressed in the canonical pattern found in arthropods: ectodermal stripes at the posterior boundary of each body segment prior to morphological segmentation in all segments regardless of tagmatization. However, it is still possible that en has a role in segment formation in Chaetopterus. Because our experiments do not directly test function, we can only implicate a role for Ch-en in segmentation by observing expression in an iterated pattern in the ectoderm or mesoderm restricted within segments. Furthermore, the expression must temporally coincide with the elaboration of individual segments. Although initial reports of en localization in the leech Helobdella (Wedeen and Weisblat, 1991) were interpreted as being similar to that observed in arthropods, recent experimental work has shed doubt on the role of en in leech segmentation and gangliogenesis (Shain et al., 1998; Seaver and Shankland, 2001). Rather, it appears that, in annelids, en orthologs are expressed in subsets of specific cell types that are themselves distributed in a segmentally iterated pattern. We would predict that Ch-en loss-of-function experiments would lead to an absence of individual cell types but not to mirror-image duplications or other radical transformations of segmental organization. It will be critical to experimentally manipulate en expression to further clarify this issue and also to examine expression in polychaete species that exhibit distinct modes of segment formation relative to Chaetopterus to understand the extent of variability in en expression among polychaetes.

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